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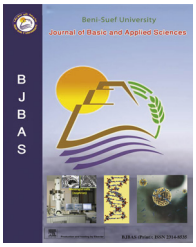

Full Length Article

Polyphenolic content and biochemical evaluation of fijk, alomo, osomo and oroki herbal mixtures *in vitro*

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ABSTRACT

Background: The current upsurge in the use of herbal remedies coupled with loose regulation on public access to these products underscore research efforts to evaluate their biochemical effect, noting also that many of the herbal medicines lack scientific credence to support medicinal claims.

Objective: Determination of *in vitro* antioxidant capacity and membrane stabilizing potential of some herbal remedies (Fijk, Osomo, Alomo and Oroki) respectively.

Methodology: Red blood cells (RBCs) were prepared from rat blood and exposed to the therapeutic doses of Fijk, Alomo, Osomo and Oroki herbals in order to estimate relative hemolysis. Distilled water treatment of RBCs was taken as 100% hemolysis. Subsequently, the hemolysates were used for the determination of glucose-6-phosphate dehydrogenase (G6PD), superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA). The herbals were further evaluated for their polyphenolic content, free radical scavenging activity as well as total antioxidant capacity.

Results: The herbals showed low polyphenolic content, reduced antioxidant capacity and offered no protection against free radical-induced degradation of deoxyribose. On the other hand, the herbal mixtures caused appreciable hemolysis of RBCs as well as depleted the levels of rat erythrocyte G6PD, GSH and SOD. Also, the erythrocyte level of MDA was elevated ($p < 0.05$) by exposure to Oroki herbal mixture.

Conclusion: The herbal mixtures have low polyphenolic content as well as poor antioxidant property. This may impede their capacity to protect against oxidative stress.

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1. Introduction

Medicinal plants are a major source of active drugs from nature. The use of plant parts in treating diseases is universal, it is often more affordable and believed to be effective than the conventional drugs. Most of these medicinal plants are eaten or used for their rich phytochemical constituents, which provide both preventive and curative properties to consumers against various diseases (Prohp and Onoagbe, 2012). In recent years, there has been increase in the popularity enjoyed by herbal remedy usually prepared by mixing various medicinal plant species (Adeyemi et al., 2012). Currently, the World Health Organization (WHO) estimated that 80% of the population in some Asian and African countries presently use herbal medicine as an alternative form of primary health care (World Health Organization (WHO), 2005). According to a report by Adisa and Fakeye (2006), the use of herbal medicines by the traditional practitioners for treatment of diseases is gaining increasing popularity especially among the rural populace in the developing countries and this remains the main stay of health care system.

In Nigeria, the last few years have witnessed an upsurge in the patronage of herbal remedies. In spite of the wide patronage enjoyed by herbal remedies, little or no empirical data exist to support medicinal claims or otherwise. Also, scientific data on safety and toxicity profiles of these herbal are in dearth (Adeyemi et al., 2012; Ekor et al., 2010).

Herbal remedy mixtures such as Yoyo Bitters, Swedish Bitters, Fijk, Osomo, Alomo, Oroki among others have become a common sight in many Nigerian homes. All of these herbals have acclaimed medicinal benefits but only few, if any, have empirical data to support medicinal claims. However, recent studies have demonstrated the need to subject some of the herbal mixtures to scientific scrutiny, at least in part to ascertain safety limits (Adeyemi et al., 2012; Ezejiofor et al., 2008; Ogbonnia et al., 2010), more so government regulation of herbal medicine is not as stringent when compared to conventional drugs. Furthermore, microbial contaminants and higher level of heavy metals which could be detrimental to human health have been demonstrated in several herbal remedies (Obi et al., 2006). All of these factors serve to fuel the imperativeness for empirical data on either the safety or toxicity margin of herbal mixtures being marketed and promoted to the Nigerian public.

Fijk, Alomo, Osomo and Oroki herbal mixtures are very popular among the Nigerian populace. Fijk herbal mixture is a combination of different herbs; *Cassia alata*, *Citrus medica var acida*, *Aloe barbaris*, *Aloe vera*, *Cassia angustifolia*. Alomo herbal mixture is an alcoholic herbal formulation comprising different herbs; *Khaya ivorensis*, *Capparis erythrocarpus*, *Lecaniodiscus cupanioides*, *Dialium guineense*, *Treculia Africana*. Osomo is also an alcoholic-based herbal formulation comprising different herbs; *Callichilai barteri*, *Pachylobus edulis*, *L. cupanioides*, *Allium sativum*, *Zingiber officinale*, *Monodora myristica*, *Khaya ivorensis*, *Piper nigrum*, *Eugenia caryophyllus*. Oroki herbal is a mixture of different herbs; *Sorghum bicolor*, *Khaya grandifoliola*, *Cassia sieberiana*, *Staudtia stipitata*, *Alstonia congensis*, *Ocimum basilicum*, *Mangifera indica*, *Cyathula prostrata*, *Securidaca longipedunculata*, *Saccharum officinarum*. However, there are no scientific data on any of these herbals whether to support medicinal claims or otherwise. To this end, the present study determined the *in vitro* antioxidant capacity and membrane stabilizing potential of some herbal remedies (Fijk, Osomo, Alomo and Oroki) respectively.

2. Materials and methods

2.1. Experimental animals

Wistar rats of weight between 150 and 170 g were obtained from the Experimental Animal Farm at the University of Ilorin, Ilorin, Nigeria and reared for 14 days in a well ventilated experimental room. The rats had free access to standard rat chow and clean water *ad libitum*. Handling of the animals was humane and consistent with the Best Practices as approved for, by the institutional committee on scientific study.

2.2. Herbal mixtures

Fijk, Alomo, Osomo and Oroki herbal mixtures were used as purchased at a Pharmacy store in Lagos, Nigeria. The herbals were used as aqueous preparation. Dose selection was premised on the manufacturer's recommendation for the consumption of 70 kg human.

2.3. Polyphenolic determination

The total polyphenol content (TPC) was determined according to the method described by the International Organization for Standardization – 14502-1 (ISO, 2005). Briefly, 1.0 ml of the diluted herbal mixture was transferred to separate tubes containing 5.0 ml of a 1/10 dilution of Folin–Ciocalteu's reagent water. Then, 4.0 ml of a sodium carbonate solution (7.5% w/v) were added. The tubes were then allowed to stand at room temperature for 60 min before absorbance at 765 nm was measured against water. The TPC was expressed as gallic acid equivalents (GAE) in mg/100 g material. The concentration of polyphenols in samples was derived from standard curve of gallic acid ranging from 10 to 100 μg/ml ($r^2 = 0.9876$).

2.4. DPPH scavenging activity

The DPPH scavenging activity was determined as previously described by Atolani et al. (2009). Briefly, 0.1 ml of herbal mixture or α-tocopherol (at different concentrations) was mixed with 2.9 ml of 0.1 mM DPPH–methanol solution. The mixture was incubated for 30 min at 25 °C in darkness, after which the decrease in absorbance at 517 nm was measured. Methanol was used as control instead of herbal mixture while blank contains methanol instead of DPPH. α-Tocopherol was used as standard.

Calculation; % activity

$$= 1 - [(\text{absorbance of sample} - \text{absorbance of blank})/(\text{absorbance of control})] \times 100$$

2.5. Deoxyribose assay

The assay was carried out using the method described by Walia et al. (2014) with slight modification. Briefly, the assay was

performed as non-site specific and site-specific. In non-site specific deoxyribose assay, 0.1 ml of EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of deoxyribose, 1 ml of herbal mixtures or gallic acid, 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of 100 μM ascorbic acid were added in sequence. The mixture was incubated at 37 °C for 1 h. One milliliter of the incubated mixture was mixed with 1 ml of 2.8% trichloroacetic acid (TCA) and 1 ml of thiobarbituric acid (TBA) in 0.025 M NaOH and heated for one hour on water bath at 80 °C to develop a pink chromogen which was measured at 532 nm using a UV/Vis spectrophotometer (Jenway, Staffordshire, UK). In site-specific deoxyribose assay, EDTA was replaced with phosphate buffer.

2.6. *Total antioxidant capacity*

The total antioxidant capacity of the herbal mixtures was determined using the phosphomolybdate method as described by Jayaprakasha et al. (2002) with little modification. α-Tocopherol was used as reference compound. Briefly, 0.3 ml of the herbal (100 mg) solution was added to tubes containing 3 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in water bath at 95 °C for 90 minutes. The samples were cooled to room temperature and the absorbance was measured at 695 nm against the blank using an UV/Vis spectrophotometer (Jenway, Staffordshire, United Kingdom). Methanol was used to replace the samples in the blank solution. The total antioxidant capacity was extrapolated from a calibration curve and expressed as μg equivalents of α-tocopherol.

2.7. *Preparation of red blood cell (RBCs)*

Blood was collected from Wistar rats into EDTA bottles. The blood was spun at 1500 *g* for 10 minutes in a refrigerated centrifuge (Anke TDL-5000B, Shanghai, China). The plasma and buffy coat were discarded. The RBCs were washed three times with phosphate buffered saline (PBS), pH 7.4 and were further spun at 1500 *g* for 10 minutes. The RBC sediment obtained was diluted to 50% using PBS and used for hemolysis estimation.

2.8. *Experimental design*

The 50% RBC was divided into six treatment groups as follows:

Control: 50% RBC + PBS (pH 7.4)
Fijk: 50% RBC + PBS (pH 7.4) + 20 mg/ml Fijk herbal
Alomo: 50% RBC + PBS (pH 7.4) + 20 mg/ml Alomo herbal
Osomo: 50% RBC + PBS (pH 7.4) + 20 mg/ml Osomo herbal
Oroki: 50% RBC + PBS (pH 7.4) + 20 mg/ml Oroki herbal
100% hemolysis: 50% RBC + PBS (pH 7.4) + autoclaved distilled water

The mixtures were incubated for 1 hour at 37 °C on a shaker.

2.9. *Estimation of relative hemolytic activity*

Hemolysis in the samples was estimated after 1 hour incubation using the procedure described by Hashim (2014) with slight modifications. The mixtures were centrifuged at 1500 *g* for 10 min. The supernatant was collected and the absorbance recorded at 540 nm using a UV/Vis spectrophotometer (Jenway, Staffordshire, United Kingdom). The red blood cells treated with autoclaved distilled water served as control and represented 100% lysis. Percentage hemolysis was calculated using the expression below. Experiments were in triplicate.

$$\text{Hemolysis \%} = \text{OD of sample} - \text{OD of blank} / \text{OD of positive control} \times 100$$

2.10. *Biochemical assays*

Further biochemical determinations were carried out on blood hemolysates after the estimation of hemolysis was done. Where applicable, a UV/Vis spectrophotometer (Jenway, Staffordshire, United Kingdom) was employed for absorbance measurement. The level of reduced glutathione level (GSH) was determined by the procedure described by Ellman (1959) with slight modification. Lipid peroxidation was assayed by measuring the formation of thiobarbituric acid reactive substances (TBARS) using the method described by Niehaus and Samuelson (1968). Superoxide dismutase (SOD) level was determined as described by Misra and Fridovich (1972). The glucose-6-phosphate dehydrogenase (G6PD) level was determined using the Randox assay kit (Crumlin, UK).

2.11. *Data analysis*

All data were presented as the mean ± SEM. Data were subjected to statistical analysis using the one-way ANOVA (GraphPad Software Inc., San Diego, CA). Differences among the group means were evaluated by Tukey's test. Mean values were considered to be statistically significant at $p < 0.05$.

3. Results

The four herbal remedies were subjected to polyphenol estimation as gallic acid equivalent (GAE). The polyphenolic contents of the herbal mixtures were less than 1% GAE (Fig. 1). For the DPPH assay, results revealed that α-tocopherol had significantly higher capacity to scavenge the free radical species compared to the four herbal mixtures (Fig. 1). The scavenging property of the herbal mixtures was only dose dependent in Fijk exposure. The herbal remedies were further evaluated for their capacity to protect against hydroxyl (OH^-) radical-induced degradation of deoxyribose using the *in vitro* Fenton-type system which included the non-site specific ($Fe^{2+} + H_2O_2 + EDTA$) and site specific ($Fe^{2+} + H_2O_2$) assays. In this assay, OH^- radicals are generated in free solution that attack the deoxyribose substrate and fragmenting it into thiobarbituric acid reactive substances (TBARS). In the non-specific site assay, the herbals had <1% scavenging activity (Fig. 2). However, in the specific site assay, Fijk herbal showed approximately 34% OH^- scavenging activity. This is compared to the gallic acid standard which had >75% inhibition of deoxyribose degradation in both site and non-site specific assays.

The phosphomolybdate assay was used to evaluate the total antioxidant capacity of the herbal mixtures. The

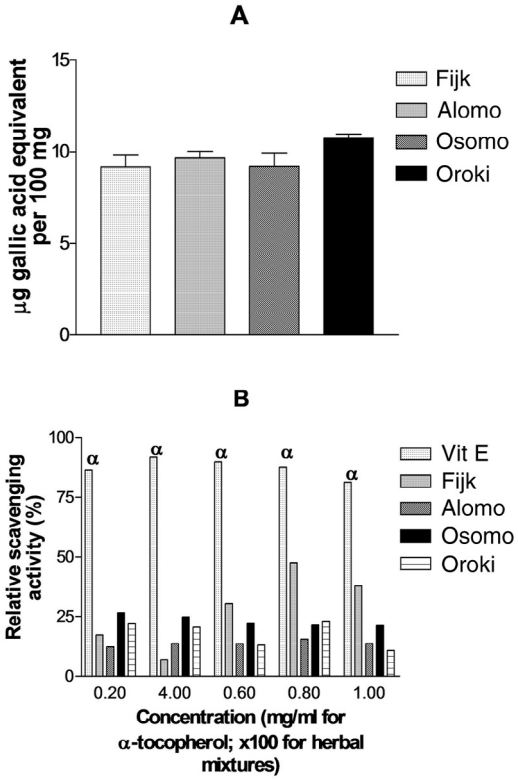

Fig. 1 – (A) Polyphenolic content in herbal mixtures (µg gallic acid equivalent/100 mg). (B) DPPH scavenging activity (%) of herbal mixtures and α-tocopherol. Data are presented as mean value ± SEM (n = 3). α = $p < 0.05$ versus control.

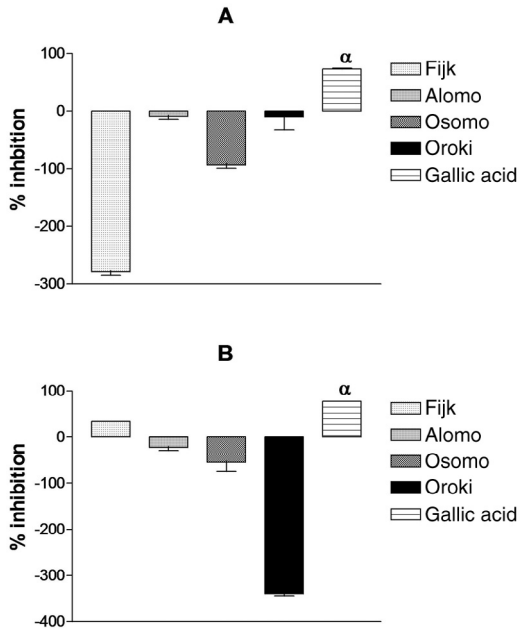

Fig. 2 – (A) Percentage inhibition of free radical induced degradation of deoxyribose by herbal mixtures and gallic acid – (Non-specific site inhibition). (B) Percentage inhibition of free radical induced degradation of deoxyribose by herbal mixtures and gallic acid – (Specific site inhibition). Data are presented as mean value ± SEM (n = 3).

phosphomolybdate method is quantitative since the total antioxidant capacity is expressed as α-tocopherol (α-tocopherol) equivalent. The herbal mixtures demonstrated low antioxidant capacity with <5 µg α-tocopherol equivalent per 100 mg.

In order to determine whether the herbal mixtures could predispose to oxidation, red blood cell (RBC) was incubated with the various herbal mixtures and the relative hemolysis was estimated. Distilled water treatment of the rat blood was taken as 100% hemolysis. The relative hemolysis caused by exposure to the four herbals was between 45 and 60% (Fig. 3).

Next, the level of G6PD in erythrocyte sediment was determined for the various treatment groups. The exposure to the four herbal mixtures significantly reduced the erythrocyte level of G6PD relative to the control (Fig. 4). In the same vein, the levels of GSH and SOD in the erythrocyte were depleted ($p < 0.05$) when compared to the control. Furthermore, estimation for evidence of lipid peroxidation showed that the exposure to Oroki herbal mixture caused elevated ($p < 0.05$) levels of MDA in erythrocyte (Fig. 5). Fijk, Alomo and Osomo did not cause any appreciable increase to the level of erythrocyte MDA.

4. Discussion

Scientific data on herbal medicine have become imperative in light of the belief that herbal products are natural and without the side effect commonly associated with conventional medicine. This often has been proven otherwise (Adeyemi et al., 2012; Joshua et al., 2010). In recent times, the Fijk, Alomo, Osomo and Oroki herbal remedies are perceived as tonic for improving health and have become highly popular among the Nigerian populace. Although there are scientific reports on the individual medicinal plant constituents of the herbal mixtures, there are no empirical data to support claims of medicinal benefits or otherwise of Fijk, Alomo, Osomo and Oroki mixtures. A search through online research databases including Cochrane, PubMed, and Google-Scholar revealed no scientific data on Fijk, Alomo, Osomo and Oroki herbal remedies. The present study affords preliminary scientific data on the *in vitro* biochemical evaluation of these herbals.

The low polyphenolic contents in Fijk, Alomo, Osomo and Oroki may implicate little contribution by these phytoconstituents to the medicinal benefits afforded by the herbals. More so, the low polyphenolic content may contribute to limit the potential of the herbals to provide the much acclaimed health promoting benefits. Studies have attributed several health-promoting effects to the presence of polyphenol contents in extracts (Adeyemi et al., 2009, 2010, 2011, 2013; Ekor et al., 2013; Fabiyi et al., 2012).

In assessing the capacity of the different herbal mixtures to scavenge free radicals, the DPPH assay showed significantly low scavenging potential for the herbal mixtures when compared to α-tocopherol. The inability of the different herbal mixtures to significantly scavenge the free radicals implicates reduced antioxidant strength. This may be related to the low polyphenolic contents in the herbals. Polyphenolic

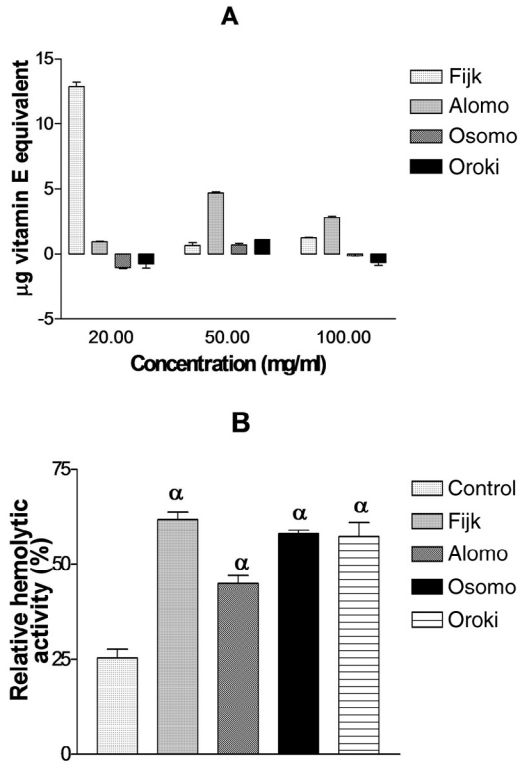

Fig. 3 – (A) Total antioxidant capacity of herbal mixtures (µg α-tocopherol equivalent/100 mg). (B) Relative hemolytic potential (%) of herbal mixtures. Data are presented as mean value ± SEM (n = 3).

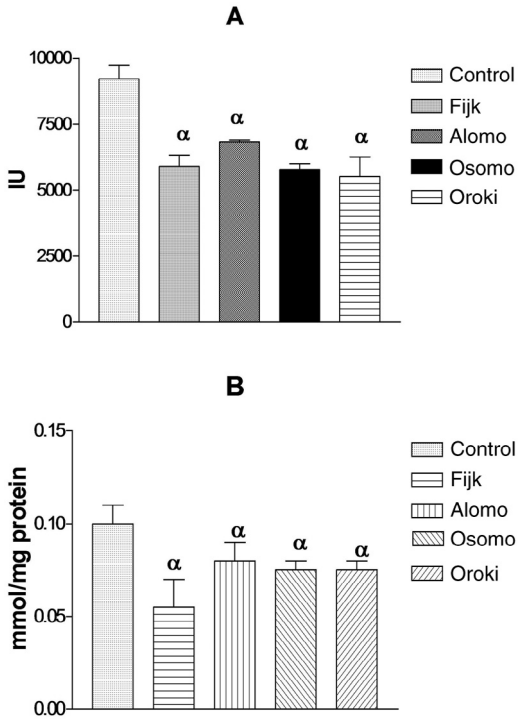

Fig. 4 – (A) Level of glucose-6-phosphate dehydrogenase in rat erythrocyte following exposure to herbal mixtures. (B) Level of reduced glutathione in rat erythrocyte following exposure to herbal mixtures. Data are presented as mean value ± SEM (n = 3). α = $p < 0.05$ versus control.

compounds have been shown to possess strong antioxidant potential against DPPH (Atolani et al., 2011). Further analysis using the Pearson correlation revealed an inverse relationship between the DPPH activity and polyphenol contents for Fijk, Alomo, Osomo and Oroki (with r values; –0.4, –0.8, –0.7, –0.5 respectively). Another probable reason for the low antioxidant capacity may be that the lipid phase in which the DDPH radical was generated limited the scavenging capacity of the herbals which are in aqueous phase as against the lipid soluble α-tocopherol. However, it would have been more desirable if the herbals could offer antioxidant protection in both the hydrophobic and hydrophilic phases.

Furthermore, the herbals showed abysmal potential in protecting against OH^--induced deoxyribose degradation. The inability of the herbals to inhibit the OH^--induced degradation of deoxyribose was a reflection of poor hydroxyl scavenging potential of the herbals. This also may be attributable to the low polyphenolic content of the herbal mixtures. Polyphenolic compounds have been shown to possess strong OH^- radical scavenging potential with protection against OH^--induced deoxyribose degradation (Walia et al., 2014). In the same manner, the total antioxidant capacity determined by phosphomolybdate method revealed low antioxidant equivalent of α-tocopherol in the herbal mixtures. The total antioxidant capacity is quantitative and reflects the potential of compound(s) to serve as antioxidants with reference to known or standard antioxidants such as α-tocopherol. The low total antioxidant capacity as revealed in this study is a representation of the poor radical scavenging potential exhibited by the herbal mixtures.

The hemolysis study showed that the herbals have membrane toxicity potential. This may suggest the presence in the herbal mixtures of ingredients that could make cellular membrane liable to instability. For instance, phytoconstituents like the saponins have been shown to cause blood hemolysis (Arias et al., 2010; Hashim, 2014). Although the present study did not determine the presence of phytoconstituents, it is probable that such hemolytic phytoconstituents form part of the herbal formulations. Normally, hemolysis results from RBC swelling due to the formation of pores and/or channels in the plasma membrane. Hemolysis can also be caused by direct membrane disintegration under detergent or ultrasonic treatments (Arias et al., 2010). The mechanism by which the herbals cause blood hemolysis is yet unknown, however, the hemolytic potential demonstrated herein is evidence that the herbals may have potential to alter membrane stability.

G6PD is the first enzyme in the pentose phosphate pathway, and physiologically, it catalyzes the production of NADPH and pentose sugars (Al-Awaida and Akash, 2014). The NADPH plays important role in erythrocytes, by preserving the integrity of red blood cell membrane sulfhydryl groups and detoxifies harmful hydrogen peroxide and oxygen radicals. Also, NADPH is used in the regeneration of reduced glutathione which prevents membrane destabilization (Abboud and Awaida, 2010; Ciftci et al., 2004). Indeed, G6PD enzyme activity can be correlated with the life time of red blood cell. In this study, levels of G6PD were determined in erythrocyte following exposure to various herbal mixtures. The herbal mixtures significantly reduced the levels of G6PD relative to control. The cause for

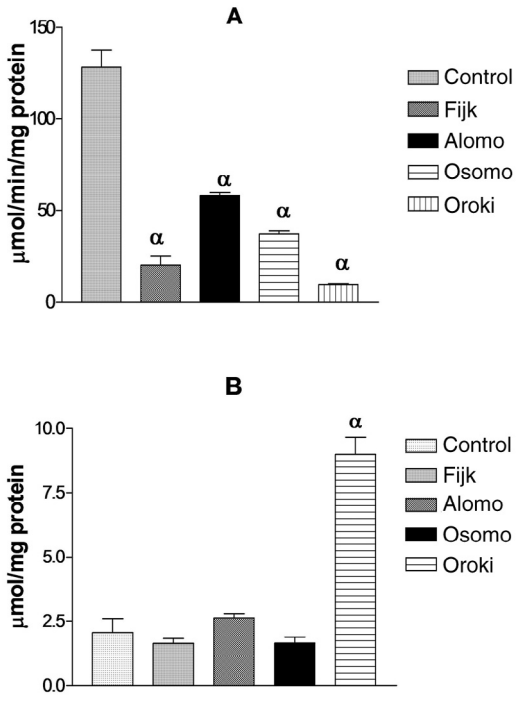

Fig. 5 – (A) Level of superoxide dismutase in rat erythrocyte following exposure to herbal mixtures. (B) Level of malondialdehyde in rat erythrocyte following exposure to herbal mixtures. Data are presented as mean value ± SEM (n = 3). α = $p < 0.05$ versus control.

the reduction of G6PD is not clear yet but probable reason may be the inactivation of G6PD by the herbal remedies. Studies have shown the potential of natural product compounds to inhibit the activity of G6PD (Mikami et al., 2013). Whether the inhibition of G6PD activity by the herbals restricts their capacity to complement antioxidant machinery in cells remains unknown.

To further evaluate the potential of the herbal mixtures to maintain the antioxidant status or otherwise of rat RBCs, the levels of GSH and SOD were determined. The herbal mixtures caused depletion in the levels of erythrocyte GSH and SOD. GSH is a non-enzymatic antioxidant molecule capable of scavenging free radicals. It also serves as substrate for other antioxidant enzymes including glutathione peroxidase. It is possible that the herbal mixtures promoted the generation of free radicals causing increased scavenging activities by the GSH molecules and hence the depletion. SOD catalyzes the dismutation of superoxide into hydrogen peroxide which may be further broken down to water by glutathione peroxidase or catalase. The level of SOD in the present study may further underscore ensuing oxidative stress. SODs are the major antioxidant enzymes that inactivate superoxide and thereby control oxidative stress (Korrea, 2007). Induction of antioxidants by oxidative stress in order to protect cells from further oxidant injury has been reported (Korrea, 2007). Several studies have demonstrated increased levels of SOD enzyme in individuals exposed to various kinds of stressors (Adeyemi et al., 2012; Elosua et al., 2003; Kanehira et al., 2006; Mak et al., 2004). Accordingly, the reduction in the level of erythrocyte SOD as seen in the present study has the potential to make cell susceptible to oxidative damage. It has been demonstrated that imbalance in antioxidant mechanisms influence cellular sensitivity to free radical damage (Ames, 1983).

In order to determine whether the herbal mixtures could potentiate lipid peroxidation, the levels of MDA were determined in erythrocyte after exposure to the herbals. Oroki herbal medicine raised the MDA levels appreciably compared to the control. This may point to ensuing oxidative stress. This could further be reinforced by depleted levels of erythrocyte GSH and SOD consequent of the herbal exposure. A previous study has reported the potential of herbal mixture to elevate MDA as well as reduce GSH levels in a manner that reflects oxidative stress (Adeyemi et al., 2012).

In conclusion, the four herbals; Fijk, Alomo, Osomo and Oroki mixtures possess poor antioxidant capacity as well as limited radical scavenging activity and these correlate well with the low polyphenolic contents of the herbals. These herbal mixtures do not have significant antioxidant activity, although many of plant species included in the composition of these herbs are proven in the literature to possess individually significant antioxidant activity. The data further show preliminary evidence implicating membrane toxicity by the herbals. Caution in the consumption of the herbals is advised.

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